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SOME KINETIC CHARACTERISTICS OF THE ENZYMATIC OXIDATION OF LYMPOXANTHINE DETECTED BY THE CHEMOLUMINESCENCE OF LUMINOL

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Pablo R. Blanco, Gladys M. Oyamburo, Eugenio Prodanov, Biochemistry Department of the School of Medicine, Montevideo, and Carlos Garcia Moreira, Biophysics Department of the School of Medicine, Montevideo

INTRODUCTION

The use of chemoluminescent reactions as detectors of oxidation-reduction reactions has made possible the development of artificial luciferine-luciferase systems and the application of these systems to the study of enzymatic reations [1, 2]. The reactions most studied by this method are the oxidation reactions of lympoxanthine catalyzed by xanthine oxidase from milk [1-3], xanthine oxidase from cow's liver [4], and xanthine dehydrogenase from chicken liver [5, 6]. The possible relationship between chemoluminescence and free radical reactions has been recently confirmed by simultaneous recordings of the emission of light and paramagnetic resonance [7].

Although the emission curves obtained by the xanthine oxidase-lympoxanthine-luminol system have characteristics of the emission curves of enzyme-substrate complex, some peculiarities of the enzymatic reaction associated with the chemoluminescent reaction offer some problems to the kinematic interpretation of the records. We are going to discuss these problems in this paper.

MATERIALS AND METHODS

a) Apparatus

The measurements of chemoluminescent emission were made with a photofluorometer "Photovolt," model 540, adapted for that purpose by Dr. W. De Angelis in our laboratory. This adaptation consists of a special addition that allows the injection of reactants into the reaction tube in the dark and under continuous oxygen bubbling. The emission was recorded on paper using a Texas recorder model R-1000. The measurements

of light emission were made by provoking the reaction with an injection of enzyme into the mixture of the other reactants, which had been previously prepared in a reaction tube and under oxygen bubbling.

The spectrophotometric measurements of velocity were made using a Beckman D. U. spectrophotometer.

b) Reactants

The xanthine oxidase was prepared from lard whey, according to the Horecker and Heppel method [8]. The sample used in the experiments described in this paper contained 11.4 mg of protein per milliliter, and its activity per milliliter of protein was 5.4 units of increment of optical density at 290 mu/minute, using the Kalckar method [9]. This enzyme was preserved frozen in buffer of phosphates, 0.5 M. pH 7.5, and samples for the experiments were taken from it. In these experiments the enzyme was used in solutions of different concentrations, which will be specified in each case in the caption of each figure. In different experiments not described in this paper, enzymatic compounds of different activities and characteristics were used, and the same results were observed.

Lympoxanthine Schwarz without further purification was used, as well as luminol (5-amino-2,3-dehydro-1,4-phathalein dione) highly purified (given by Professor J. R. Totter's laboratory (Athens, Georgia, USA)).

c) Procedures

In the reactions of the enzymatic chemoluminescent system, the intensity of emission as a function of time was recorded, and these records served to study (1) the maximum emission intensity, and (2) the characteristics of the emission curves in the decreasing region.

The emission curves were studied as functions of the variables enzyme concentration (E), initial substrate concentration (S)in and pH.

In additional experiments, the effect of other factors on certain characteristics of the emission curves was explored.

In the case of the reactions studied by means of the spectrophotometer, the production of uric acid was detected by the increase in optical density to 290 mu.

RESULTS

a) Characteristics of the Emission Curves

Although the obtained curves look like the curves produced by the enzyme-substrate complex [4], a special behavior is detected when the initial concentration of substrate is varied. It is peculiar that, in the individual curves, the zone of constant emission expected in a kinetic situation of zero order for large initial concentrations of substrate was not observed. The individual curves reach a maximum intensity which is immediately followed by a decreasing portion. This portion becomes longer

as the initial concentration of lympoxanthine increases. This phenomenon is illustrated by the family of curves in Figure 1.

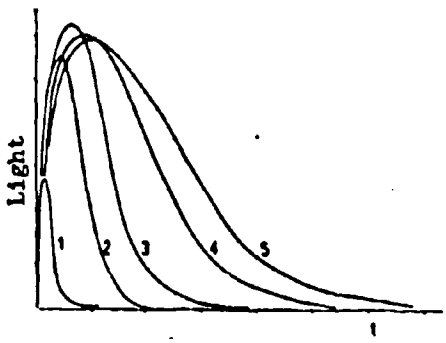

Figure 1. Series of emission curves produced by the xanthine-oxidase-lympoxanthine system in the presence of luminol. Concentration of the reactants: Lympoxanthine, 3.1, 9.3, 15.6, 26.1 and 40.6 x 10^{-5} M. (curves I through V respectively). Luminol 5 x 10^{-4} M. Buffer of carbonate bicarbonate pH 9.2, concentration 2.5 x 10^{-2} M. Activity of enzyme specified in the text, with a total dilution of 1/160.

Such facts presented the challenge of measuring and interpreting this phenomenon. In order to do this, the decreasing part of the emission curves was adjusted to kinetic zones of the first order, which is justified experimentally and allows to define a constant characteristic of each curve. According to this approximation, and based on the hypothesis that the values of emission intensity at every moment are a measure of the concentration of the enzyme-substrate complex (ES), it is possible to write the following differential equations:

$$\frac{d(ES)}{dt} = k(ES) \qquad (1)$$

and

$$\frac{d(ES)/dt}{(ES)} = k \qquad (2)$$

where (ES) represents the concentration of enzyme-substrate complex (measured by the intensity of emission), t represents the time and k is the first order constant characteristic of the decreasing part of the curve. This relation was confirmed experimentally in the three groups of experiments related to the variables (E), $(S)_{in}$ and pH. In each case the equation $d(ES)/dt = a + k(ES)$ was adjusted by the method of the least squares in the interval of relative (ES). The accuracy of the adjustment was verified by calculating z (Fisher's proof of linearity), the values obtained for z are not significant, not even in the significant level corresponding to 5%.

b) Characteristic Modifications of the Curves as Functions of the Variables (E), (S) and pH

As shown in Figure 2, the values of the constant k are directly proportional to the enzyme concentrations within the studied interval of

concentration. The adjusted line intersects the axis of ordinates near the origin.

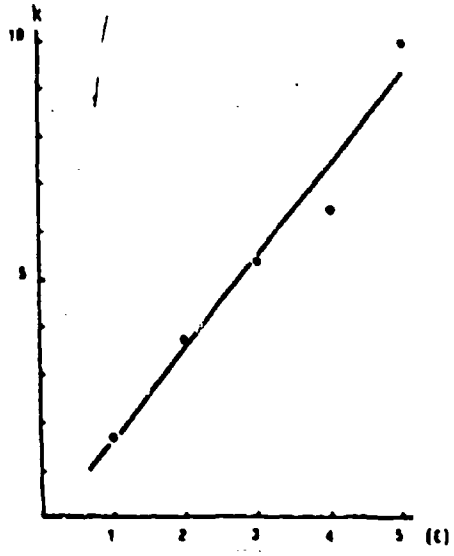

Figure 2. Variation of the first order constant in the last portion of the emission curves as a function of the enzyme concentration. Concentration of reactants: Lympoxanthine 10^{-4} M., buffer of carbonato-bicarbonate pH 9.7, concentration 2.5 x 10^{-2} M. (E): enzyme concentrations x 1/320. Values of k in $min.^{-1}$. Temperature 17°C.

The curve showing the variation of K as a function of the initial (S), (Figure 3) has the aspect of a parabola, one of whose branches intersects the axis of ordinates, (maximum value of k when $(S)_{in}$ tends to zero) while the other branch approaches the axis of abscissas asymptotically, (k tends to zero when $(S)_{in}$ tends to infinity). These relations come from the graphs in Figures 4 and 5, which are based on the same series of experiments as Figure 3. From them it can be seen that the relation between k and $(S)_{in}$ corresponds to a function of the type $k = a/[(S)_{in} + b]$, where k is the constant defined in this paper, $(S)_{in}$ represents the initial concentration of substrate, a and b are two indeterminate constants.

All other conditions being constant, the increase in pH between 9.7 and 10.4 produces a decrease in k, as can be observed in Table 1. At relatively high pH's (10.4, for example), a smaller effect of the initial concentration of substrate on the value of the constants is observed, but the maximum value of k always corresponds to the smallest initial concentration of lympoxanthine.

c) Other Factors Modifying the Emission

The constant defined in this paper is independent of the intensity of emission when the latter is affected by factors or reactants which do not affect the enzymatic activity in the same degree. Different reactants, like cyanide, menadione, oxygen and uric acid, appreciably affect the emission intensity without affecting the enzymatic activity in the same degree. The effect of these reactants on the constant k is generally parallel to

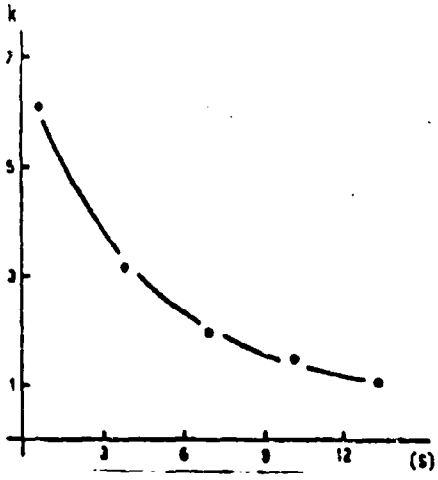

Figure 3. Variation of k as a function of the initial concentration of reactants. Lympoxanthine: 0.625, 3.75, 6.90, 10.0 and 13.10 x 10^{-5} M. Luminol 5 x 10^{-4} M. Buffer of carbonate-bicarbonate pH 9.7, concentration 2.5 x 10^{-2} M. Enzyme diluted to 1/160. (S): initial concentration of substrate (lympoxanthine); k in $min.^{-1}$. Temperature 19°C.

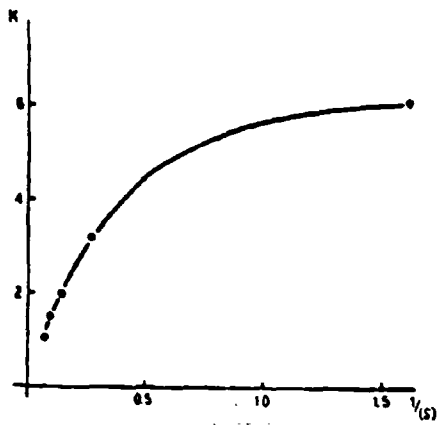

Figure 4. Same conditions as in Figures 3 and 5.

the effect they cause on the enzymatic activity measured by the production of uric acid, and independent of the effects produced on the emission intensity. Both effects are frequently opposed. These facts will originate other reports.

DISCUSSION

The fact that the first order constant of the final zone of the emission curves is proportional to the enzyme concentration is particularly interesting since the factors affecting the measurements of emission intensity are many. The effects that certain reactants or certain conditions

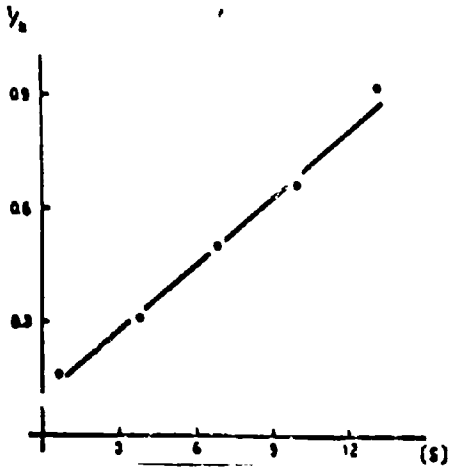

Figure 5. Same conditions as in Figures 3 and 4.

Table 1. Values of the Constants of Decrease of (ES) as Functions of the Variables (S) and pH

pH:	9.7	9.8	10.0	10.4	10.8
(S)		Values of k ($min.^{-1}$)			
0.625	7.37	6.94	3.99	--	1.29
3.75	3.06	1.20	0.91	0.53	0.40
6.90	1.39	0.91	0.68	0.30	0.38
10.00	1.27	0.68	0.59	0.32	0.27
13.00	0.84	0.72	0.65	0.42	0.15

The values of (S) (initial concentration of substrate) are given in moles x 10^{-5}.

could have on the emission intensity without affecting the enzymatic reaction would be eliminated by determining k. Supposing that the emission intensity is proportional to the concentration of enzyme-substrate complex, k would be given by equation (2) and the factors affecting the measurement of (ES) will not affect the value of the constant. The fact that we ignore the molar concentration of (ES) will not affect the value of k either.

At the point where an emission intensity curve reaches a maximum, d(ES)/dt = 0, therefore, k = 0. At this point the characteristics applicable to the stationary state and its kinematic derivations are valid, which justifies taking into account the value of v, measured as emission intensity, for the calculation of Km, according to the classical equation of Michaelis [10]. Since this maximum intensity is reached almost instantaneously for low initial concentrations of substrate, it can be said that the (S) corresponding to the maximum intensity differs very little from the initial (S).

Under conditions of non-stationary state as in the final portion of the emission curve, we have:

$$k = \frac{d(ES)/dt}{(ES)} = k_1 \frac{(E)(S)}{(ES)} - k_1(S) - (k_2 + k_3) \neq 0 \qquad (3)$$

where k_1 represents the velocity constant of the bimolecular reaction of (E) and (S) to form (ES), k_2 is the velocity constant of the inverse reaction and k_3 is the velocity constant of decomposition of (ES), yielding the products and giving back the free enzyme. The fact that this expression approaches a constant for sufficiently low values of (S) (final portion of the (ES) curve) could be explained in the following ways:

1) The value of $k_1(S)$ is very small in comparison with that of $-(k_2 + k_3)$ for very low concentrations of (S).

2) As for $k_1(S)(E)/(ES)$, its value decreases as (S) decreases, but at a slower rate than $-k_1(S)$, since its variation is determined by the ratio (S)/(ES). Its variation becomes smaller as the end of the reaction approaches.

From equation (3) it can be deduced that k must vary in the same way as (E) if the reaction is not of zero order. As for the effect of (S) initial on the value of k, the fact that k decreases as $(S)_{in}$ increases cannot be explained from equation (3). The apparent effect is that an increase of (S) initial determines a decrease in enzyme activity in this system. This fact becomes evident comparing Figure 1 with the curves described by Chance [11] for the variation of (ES) according to the kinetics of Briggs-Haldane. This leads to the conclusion that the reaction of xanthine-oxidase with lympoxanthine detected by the chemoluminescence of luminol does not follow this kind of kinetics.

Neither is the observed phenomenon caused by the classic inhibition due to excess of substrate, since this excess is not observed in this range of concentrations, or by the spectrophotometric measurements of velocity or by the maximum emission intensities (Figure 6).

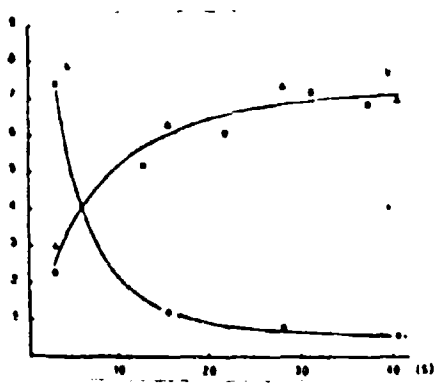

Figure 6. Graph comparing the variation of the velocities v and the first order constants k, as functions of the initial concentration of substrate (S). Ordinates: values of k and v. ■: values of v in variation of optical density per minute at 290 mu x 10^{-2}. Δ: values of maximum intensity of chemoluminescent emission in arbitrary units. 0: values of k in $min.^{-1}$. Enzyme diluted to 1/100. Temperature 26°C. Other conditions are the same as in previous graphs.

SUMMARY

A study is carried out of the curves of luminescence by reaction of xanthine oxidase with hypoxanthine in the presence of luminol and oxygen.

A final decreasing portion with a first order kinetics, is described, a first order constant, k, being defined for this final portion.

This constant is studied in relation with the parameters enzyme concentration, initial concentration of substrate and pH.

Within the range of concentrations considered, the values of k are directly proportional to the concentration of enzyme, which enables assessment of its activity independently of the intensity of emission.

As to the influence of the initial concentration of substrate, results disagreeing with the kinetics of Briggs-Haldane being described.

BIBLIOGRAPHY

1. Totter, J. R., Scosseria, J. L , and Medina, V. J., Sistemas artificiales luciferina-luciferasa, An. Fac. Med. Montevideo, 44:463-468, 1959.

2. Calcagno, M., and Prodanov, E., Estudio de la glucosa-oxidasa por medio de reacciones quemoluminicentes, Actas del VIII Congreso Latinoamericano de Quimica, Buenos Aires, 119-120, 1962.

3. Totter, J. R., Medina, V. J., and Scosseria, J. L., "Luminescence During Oxidation of Hypoxanthines by Xanthine Oxidase in the Presence of Dimethylbiacridylium Nitrate," J. Biol. Chem., 235:238-241, 1960.

4. Totter, J. R., Castro de Dugros, E., and Riveiro, C., "The Use of Chemoluminescent Compounds as Possible Indicators of Radical Production During the Xanthine Oxidase Action," J. Biol. Chem., 235:1839-1842, 1960.

5. De Angelis, W., and Totter, J. R., "Kinetics of Xanthinedehydrogenase-dimethylbiacridylium Ion Hydrogen Transfers," Fed. Proc. (Abstracts), 22:467, 1963.

6. De Angelis, W., and Totter, J. R., "Chemoluminescence Induced by Xanthinedehydrogenase," Fed. Proc. (Abstracts), 21:52, 1962.

7. De Angelis, W. (Personal communication).

8. Horecker, B. L., and Heppel, L. A., "Xanthine Oxidase From Milk," in Methods in Enzymology, S. P. Colowick and N. O. Kaplan Edit., Vol. II, p. 482, Academic Press, New York, 1955.

9. Kalckar, H. M., "Differential Spectrophotometry of Purine Compounds by Means of Specific Enzymes," J. Biol. Chem., 167:429-443, 1947.

10. Michaelis, L., and Menten, M. L., Die Kinetic der Invertinwirkung, Biochemische Zeitschrift, 49:333-369, 1913.

11. Chance, B., "The Kinetics of the Enzyme-Substrate Compounds of Peroxidase," J. Biol. Chem., 151:553-577, 1943.